EP/04/7617

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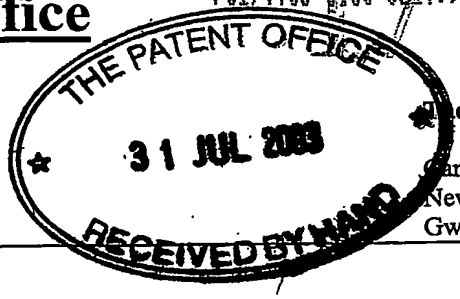

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1.	Your reference	LB1826
2.	Patent application number *(The Patent Office will fill in this part)*	0317999.1
		31 JUL 2003
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	Université of Liege Quai van Beneden, 25 4020 Liege Belgium
	Patents ADP number *(if you know it)*	
	If the applicant is a corporate body, give the country/state of its incorporation	Belgium
4.	Title of the invention	Improvements in or relating to drug delivery systems
5.	Name of your agent *(if you have one)*	Barker Brettell
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	10-12 Priests Bridge LONDON SW15 5JE
	Patents ADP number *(if you know it)*	7442494003

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of Filing *(day/month/year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing *(day/month/year)*

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Description	11
Claim(s)	
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Priority documents	
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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
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11. I/We request the grant of a patent on the basis of this application.

Signature Barker Brettell Date

Barker Brettell 31 July 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

Lance Butler Tel: 020 8392 2234

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IMPROVEMENTS IN OR RELATING TO DRUG DELIVERY SYSTEMS

FIELD OF THE INVENTION

This invention concerns improvements in or relating to drug delivery systems and has particular reference to genital drug delivery systems.

BACKGROUND TO THE INVENTION

There is now accumulating evidence that epithelial cells may influence immune reactions in squamous mucosa through the production of cytokines. Granulocyte Macrophage - Colony Stimulating Factor (GM-CSF) produced by keratinocytes acts as a selective chemoattractive molecule for the migration of dendritic cells (DC) into the epithelium and for their differentiation into Langerhans cells (LC) (Kaplan et al, 1992; Pastore et al, 1997). Exposure of LC/DC to GM-CSF *in vitro* prolongs their survival and increases their capacity to present antigens to lymphocytes (Koch et al, 1990). Moreover, *in vivo*, a correlation has been observed between the amount of GM-CSF produced by some carcinomas and the distribution/differentiation of tumor-associated DC (Colasante et al, 1995). We have previously demonstrated, by using the organotypic raft culture system, that GM-CSF is a potent factor for enhancing the colonization of LC/DC in a (pre)neoplastic epithelium formed in vitro (Hubert et al, 1999). Moreover, the infiltration of organotypic cultures by DC specifically induced the apoptosis of keratinocytes transformed by human papillomavirus (HPV) whereas DC were not affected (Hubert et al, 2001).

These data suggest that the recruitment of DC into a virus-infected and/or (pre)neoplastic epithelium might be beneficial not only by stimulating anti-viral and anti-tumor immune responses but also by inducing the death of virus-infected and transformed cells.

Morbidity and mortality caused by HPV infection is a major health problem in developing countries and in the industrialised world, where direct and indirect costs from disease are very high. HPVs are common sexually transmitted pathogens inducing a spectrum of diseases ranging from benign genital warts to invasive carcinoma. Some types of HPV have been shown to be directly involved in the malignant transformation process, especially in the uterine cervix. Up to 99 percent of cervical cancer and its precursors (squamous intraepithelial lesions; SIL) may be attributed to infection by oncogenic human papillomavirus (HPV)(Bosch et al, 1995). However, HPV alone is not sufficient for tumor progression (Herrington, 1995). The role of the intrinsic immunity in controlling HPV infection and the subsequent development of SIL is shown indirectly by the increased frequency of HPV-associated lesions in patients with depressed cell-mediated immunity (Petry et al, 1994; Ellerbrock et al, 2000). Although viral antigens are expressed in a majority of preneoplastic lesions, progression to invasive cancer may occur, suggesting the existence of some qualitative an/or quantitative perturbations in the antigenic presentation function . This hypothesis is reinforced by the observation that most genital warts and SILs are characterized by a decreased density and function of LC compared to the normal paired squamous epithelium (Giannini et al, 2002; McArdle and Muller, 1986; Morelli et al, 1994). Since the immune system clearly plays an important role in influencing the natural history of the disease, there is some evidence that immune response modifiers may have therapeutic value for these lesions. Because of the central role of DC/LC in the induction and regulation of cellular immune responses, pharmacological agents that modulate the recruitment and function of these cells might have clinical interest.

The current study was designed to evaluate the capacity of several pharmaceutical formulations to topically deliver GM-CSF on HPV-associated lesions.

One major problem associated with the genital drug delivery is that the physiological conditions imposed by the protective mechanisms of the cervix or the vagina often lead to a limited contact time of administered drugs with the mucosa and a short duration of therapeutic efficacy, making a frequent dosing regimen necessary. (Robinson and Bologna, 1994; Valenta et al, 2001). The patients are known to tolerate gels better than other conventional dosage forms sush as inserts or ointments. Some bioadhesive polymers have attracted considerable attention for their opportunity to prolong the contact of drug with a mucosal surface, without inducing adverse local effects on the epithelium (Richardson at al, 1996).

SUMMARY OF THE INVENTION

In this study, we investigated the properties of hydroxypropylcellulose, polycarbophil, carbomer and poloxamer polymers. Cellulose derivatives have been widely used as thickening agents in cervico-vaginal formulations (Gauger, 1983; Lynas, 1980; MacKenzie et al, 1979). Carbopols and polycarbophils are acrylic acid polymers which have good bioadhesive properties and prolonged retention of the formulation at the site of administration (Knuth et al, 1993). Poloxamer, a block copolymer made of polyoxyethylene and polyoxypropylene, is also known for its excellent compatibility. It forms in situ a gelling liquid which has been recognized as a convenient dosage form of topical application. The liquid applied to topical area turns into gel with an increase in temperature.(Chang et al, 2002).

After incorporation of GM-CSF into these hydrogels, the different formulations were evaluated for their bioactivity after storage at 4 or 37°C and for their potential to recruit DC in organotypic cultures of HPV-transformed keratinocytes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Proliferation response of TF-1 cell line after incubation with GM-CSF extracted at different time intervals from the hydrogels stored at 4°C or at 37°C. Growth was measured by 3H-dTR incorporation. Each point shows the mean of 6 replicates and bars represent standard error.

Figure 2: Typical dendritic morphology of adherent human PBMC after culture with GM-CSF and IL-4 (May-Grumwald-Giemsa stain; magnification 200 x)

Figure 3: Phenotypical FACS analysis of adherent human PBMC after culture with GM-CSF and IL-4. Results are displayed as dot plots. All studies were performed with isotype controls and negativity limits were set according to their location.

Figure 4: Detection of DC pre-labelled with a fluorescent red probe in organotypic cultures of HPV-transformed keratinocytes (SiHa) in the absence (A) or in the presence of GM-CSF included (C) or not (B) into a polycarbophil gel at pH 6.9. Nuclei of keratinocytes are labelled in blue after DAPI staining.

DETAILED DESCRIPTION OF THE INVENTION

Analysis of the GM-CSF bioactivity following incorporation into different polymers at 4°C and 37°C using a bioassay with TF-1 cells

The GM-CSF biological activity after incorporation into the different hydrogels was measured at different intervals of time using a bioassay of growth stimulation with the GM-CSF-dependant TF-1 cell line. The injectable solution (Leucomax, Novartis, Brussels, Belgium) was chosen as positive control. The four polymers were tested at a pH value of 6.9 which was reported to be optimal for GM-CSF stability and which correspond to the pH value of the injectable solution (data from Novartis). As polycarbophil was found to be a particularly convenient

polymer for cervico-vaginal formulations and as it is usually recommended to develop cervico-vaginal formulations at acidic pH values, the polycarbophil hydrogel was also tested at pH 5.5.

As shown in Figure 1, there was a decrease in the bioactivity of the injectable solution of GM-CSF more important at 37°C than at 4°C which is in agreement with previous data (Pettit et al, 1997). We also observed an important reduction of GM-CSF activity after incorporation in the hydroxypropylcellulose (Klucel) or in the poloxamer hydrogel (Lutrol) at both temperatures. In contrast, polycarbophil gel (Noveon) pH 5.5 seemed to induce a protection against GM-CSF degradation. However, this protective effect is not observed at pH 6.9. Carbomer (Carbopol) conserved an activity of GM-CSF similar to that observed with the injectable solution at both temperatures and during at least a week.

These results suggest that the polycarbophil (Noveon) at pH 5.5 could be convenient to topically applicate GM-CSF on a squamous mucosa.

GM-CSF incorporated in the polycarbophil gel stimulates the recruitement of monocyte-derived DC in organotypic cultures of HPV-transformed keratinocytes in a similar manner as GM-CSF in solution

We investigated whether GM-CSF included in a polymer can modulate the ability of DC to infiltrate an *in vitro*-formed (pre)neoplastic epithelium, reminiscent of high-grade (pre)neoplastic lesions observed in vivo. Cells from the plastic adherent fraction of human peripheral blood mononuclear cells cultivated in the presence of 800 U/ml of GM-CSF and 20 U/ml of IL-4 for 7 days showed a morphology of DC with abundant dendritic membrane protrusions (Figure 2). By FACS analysis, these cells also displayed typical phenotypical features of DC (Figure 3) with the presence of CD1a, CD4, CD86 and CD54 (ICAM-1) antigens, a moderate expression of class II MHC (HLA-DR) molecules and the

absence of macrophage (CD14), B (CD20) and T (CD3) cell markers. DC were layered on top of organotypic cultures of HPV-transformed keratinocytes in the presence of GM-CSF incorporated or not in the polymer. The effect of GM-CSF on DC infiltration was assessed by examining cryosections of organotypic cultures frozen 48 hours after GM-CSF addition. The ability of the GM-CSF gel to modulate the infiltration of DC was determined by evaluating the number of fluorescent DC migrating into the organotypic cultures. Figure 4 illustrates representative experiments showing the density of fluorescein-labeled DC in HPV-transformed keratinocyte organotypic cultures incubated with or without exogenous GM-CSF incorporated or not in the polycarbophil gel. DC layered onto organotypic cultures of SiHa poorly infiltrated the epithelial cell layers in the absence of GM-CSF (Fig 4A), whereas GM-CSF in solution or incorporated in the gel caused a significant increase in the density of DC observed within the epithelial sheet (Fig 4B-C).

Different mucoadhesive drug delivery systems based on hydrogels have been recently developed (Eouani et al, 2001; Peppas and Sahlin, 1996; Shawesh et al, 2002). Their ability to interact with mucus glycoproteins and to remain localized to a specific site might help to deliver molecules important for the anti-viral or anti-tumor immune responses. We have previously shown that GM-CSF increases the ability of monocyte-derived dendritic cells (DC) to infiltrate a (pre)neoplastic epithelium formed *in vitro*, suggesting that the effect of a treatment based on the local administration of GM-CSF might be a recruitment into the dysplastic epithelium of Langerhans cells (LC) (Hubert et al, 1999). LC are the main professional antigen-presenting cells in squamous mucosa. These cells are able to capture viral or tumor antigens and, after migration in the draining lymph nodes, to present these antigens to T cells. Subsequently, the sensitized T lymphocytes could reach the squamous epithelium and kill the cells bearing the antigens at the origin of the sensitization.

In this study, we analyzed the stability and bioactivity of GM-CSF in different formulations of hydrogels. As the quantitative and functional deficit of Langerhans cells is a constant immune alteration in genital HPV-associated lesions (Viac et al, 1993), we used the organotypic culture system of HPV-transformed keratinocytes (Delvenne et al, 1995; Jacobs et al, 1998; Hubert et al, 1999; Delvenne et al, 2001, Renard et al, 2002).

We first demonstrated that polycarbophil gel at pH 5.5 conserves the bioactivity of GM-CSF at 4 or 37°C for at least 7 days, whereas a decrease in the biological activity of GM-CSF was observed mainly after incorporation in the hydroxypropylcellulose or in the poloxamer hydrogels at both temperatures. This could be due to interactions with the polymers which are present in the formulations at a high concentration in order to achieve the suitable viscosity.

The residual bioactivity of GM-CSF incorporated into both gels at pH 6.9 was surprisingly different. These data could be attributed to the carboxyl group present in the polycarbophil gel and absent in the hydroxypropylcellulose and poloxamer formulations. Indeed, similar results were reported with carboxylic acid containing polymers and carbomers which were shown to inhibit the degradation of insulin and other peptide drugs (Bai et al, 1996). The GM-CSF bioactivity evaluated after storage in the polycarbophil hydrogel formulated at a pH value of 5.5 was better than that observed for GM-CSF in solution. Moreover, the fact that the pH value must be decreased in order to observe the protective effect of the polymer is completely compatible with the physiological cervico-vaginal pH.

Since the bioassay with TF-1 cells required the extraction of the GM-CSF from the different hydrogels, the bioactivity detected was that of solutions of GM-CSF. It was, therefore, important to determine whether the gel affected or not the activity of GM-CSF. Indeed, due to their viscosity, the hydrogels could interfere with the diffusion and the biodisponibility of the GM-CSF. We have

demonstrated that GM-CSF included in the polycarbophil gel at pH 5.5 stimulated the infiltration of DC into an in vitro equivalent of HPV-associated (pre)neoplastic epithelium, suggesting that this hydrogel does not impair the protein release in vitro.

All together, these results suggest that the polycarbophil gel (Noveon) is compatible with the diffusion of bioactive GM-CSF with the advantage to stabilize the protein. Moreover, GM-CSF included in this plateform is also able to recruit DC into HPV-transformed (pre)neoplastic epithelial sheets. This formulation which is recommended for genital application might restore some immune functions which have been shown to be altered in HPV-associated lesions.

Examples of Materials and Methods used in accordance with the invention.

Preparation of the hydrogels

Poloxamer P407 (Lutrol) was supplied from BASF, Germany. Carbomer (Carbopol 974P) and Polycarbophil (Noveon AA1) were a gift from Noveon, Belgium. Hydroxypropyl cellulose (Klucel GF) was obtained from Herkules. GM-CSF was received from Novartis (Brussels, Belgium). All other chemicals were of analytical grade.

- *Preparation of carbopol gel :*

The gel (1.3% w/w) was prepared by dispersing the carbopol resin in purified water. The mixture was stirred until thickening occurred and then neutralized by

dropwise addition of 40% (w/w) tromethamol, until a transparent gel appeared. Quantity of tromethamol was adjusted to achieve gel pH 6.9.

- *Preparation of polycarbophil gel*

The gel (1.0% w/w) was prepared by dispersing the Noveon AA1 in purified water. The mixture was stirred until thickening occurred and then neutralized by dropwise addition of 40% (w/w) tromethamol, until a transparent gel appeared. Quantity of tromethamol was adjusted to achieve gel pH 6.9 or 5.5

- *Preparation of poloxamer gel:*

The poloxamer gel was prepared by using the cold method. Poloxamer P407 20%(w/w) was slowly added under gentle mixing to a pH 6.9 phospate buffer 0.05M at a temperature of 4°C . The mixture was allowed to dissolve overnight at 4°C until a clear solution was obtained.

- *Preparation of the hydroxypropylcellulose gels* :

Hydroxypropylcellulose 7 %(w/w) was slowly added to a pH 6.9 phospate buffer 0.05M under gentle mixing until a transparent gel appeared

Incorporation and extraction of GM-CSF into the different polymers

900 pg of GM-CSF, diluted in RPMI with 2% FCS, were incorporated per gram of different gels by gently mixing. The gels were kept for different periods of time at obscurity and at 37°C or 4°C. After 0, 24, 48, 72 and 163 hours, the gels were diluted with 6 ml of RPMI 2% FCS in order to extract the GM-CSF. After a filtration on 0.45 μm pore filters (Millex-HV, Millipore, Bedford, MA), solutions were stored at -20°C before the bioassay. GM-CSF in solution was also kept during the different intervals of time at 37°C and 4°C and filtered before storage.

Bioassay using TF-1 cell line

The factor-dependent human cell line TF-1 (ATCC, CRL-2003) was cultivated following previously published recommendations (Kitamura et al, 1989) with minor modifications. Briefly, the cells were incubated in RPMI 1640 medium containing 10% fetal bovine serum and 5 ng/ml GM-CSF at 37°C 5% CO_2. Cells were deprived of GM-CSF for 24 hours and were cultured in the presence of 2% FCS. The cells were plated in a round-bottom 96 well microtiterplate at 50,000 cells per well, in 100 μl RPMI 2% FCS and 100 μl of GM-CSF included in the different polymers for various intervals of time. The tests were performed in quintuplate. The cultures were incubated for 24 hours in the presence of ^{3}H-TdR (2 μCi/well, 7 Ci/mmol, Moravek Biochemicals, CA). Cells were harvested by an automated sample harvester (Packard, Canberra, Tilburg, The Netherlands) and counted in a liquid scintillation counter (Top Count, Packard, Canberra).

Dendritic cell cultures and labeling with lipophilic fluorescent cell tracer.

DC were generated from the adherent fraction of human PBMC with 800 U/ml GM-CSF (Novartis, Brussels) and 20 U/ml IL-4 (Biosource Europe, Nivelles, Belgium), as previously described (Hubert et al, 1998). DC generated for this study constituted a 90% pure cell population based on several criteria including morphology, forward-scatter and side-scatter values observed by FACS analysis and surface phenotype ($CD1a^+$, $CD14^-$, $CD4^+$, $CD54^+$, ...).

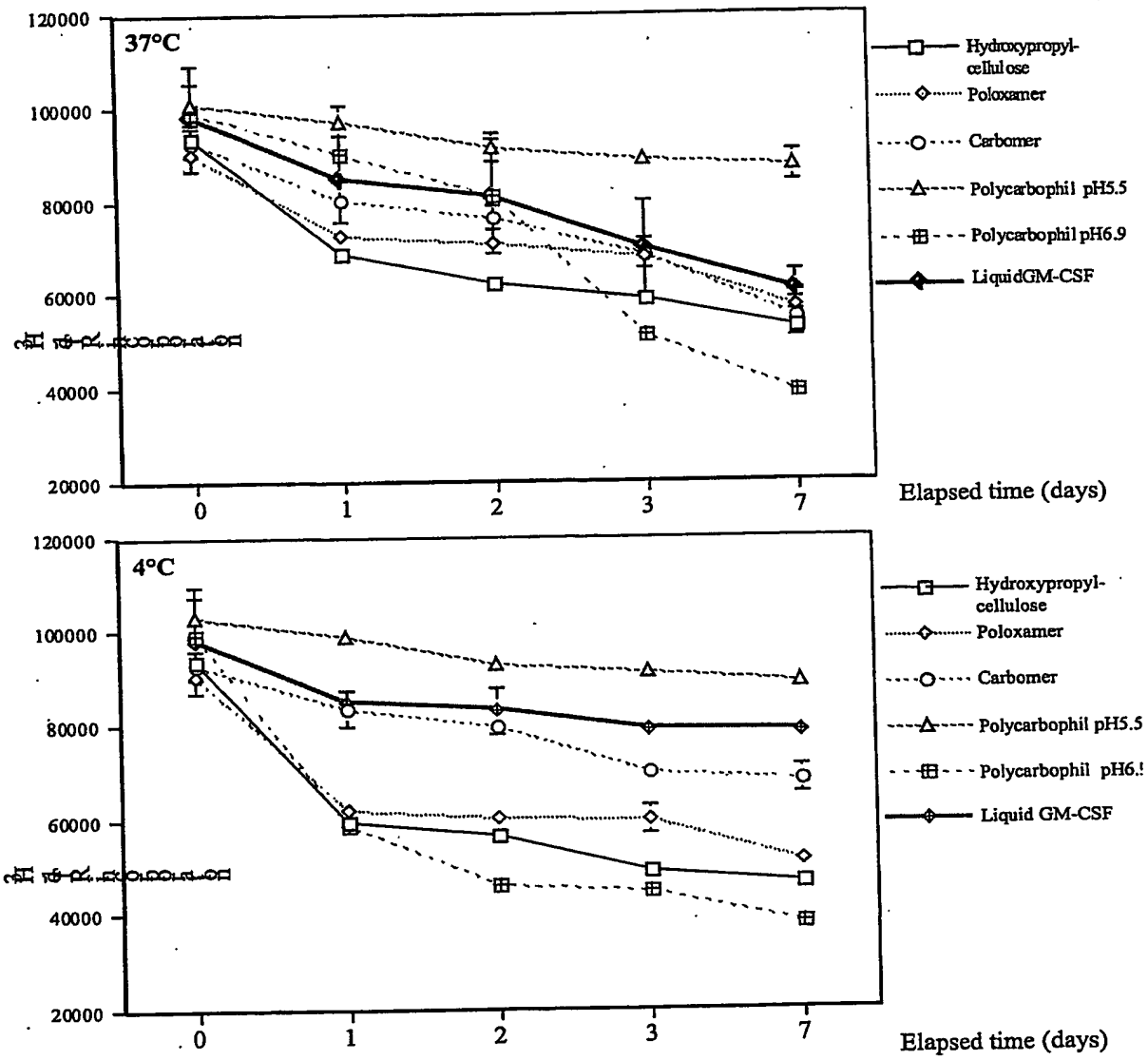
The DC were labeled with a lipophilic fluorescent marker (CM-DiL, Molecular Probes, Leiden, The Netherlands) according to a previously described procedure with minor modifications. Briefly, 4 x 10^6 DC were resuspended in 1 ml of PBS and heated to 37°C. CM-DiL was diluted in 1 ml of PBS preheated to 37°C to obtain a final concentration of 16 □g/ml. The dye was mixed for several seconds until the dye was evenly distributed. This solution was immediately transferred to

the cell suspension and rapidly mixed by pipetting. The cells were incubated for 2 min at 37°C, then for 2 min on ice. Finally, they were transferred in 40 ml of PBS at 4°C, centrifuged and resuspended in the appropriate medium.

Organotypic cultures

Organotypic cultures of HPV-transformed keratinocytes (SiHa cell line, Friedl et al. 1970) were prepared as previously reported (Hubert et al, 1999). After a 2 week stratification of keratinocytes, labeled DC were seeded on top of the *in vitro* formed epithelium at a concentration of $2x10^5$ cells/50 µl of growth medium. The organotypic culture was then placed on the different gels containing or not 8000U/ml of GM-CSF. The positive control was a solution of liquid GM-CSF at 8000U/ml After 48 H at 37°C, the collagen rafts were harvested. The cultures were then embedded in O.C.T. compound (Tissue Tek, Sakura, Netherlands) at - 70°C and sectioned with a cryostat. The organotypic cultures were counterstained with the fluorescent dye 4', 6-Diamidine-2-phenylindole Dihydrochloride (DAPI, Roche Diagnostics, Brussels, Belgium). Infiltration of fluorescent DC was visualized with a fluorescent microscope (Leica DMLB microscope, Heidelberg GmBH, Germany) equipped with a 40x objective.

Figure 1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Liquid GM-CSF

Figure 2

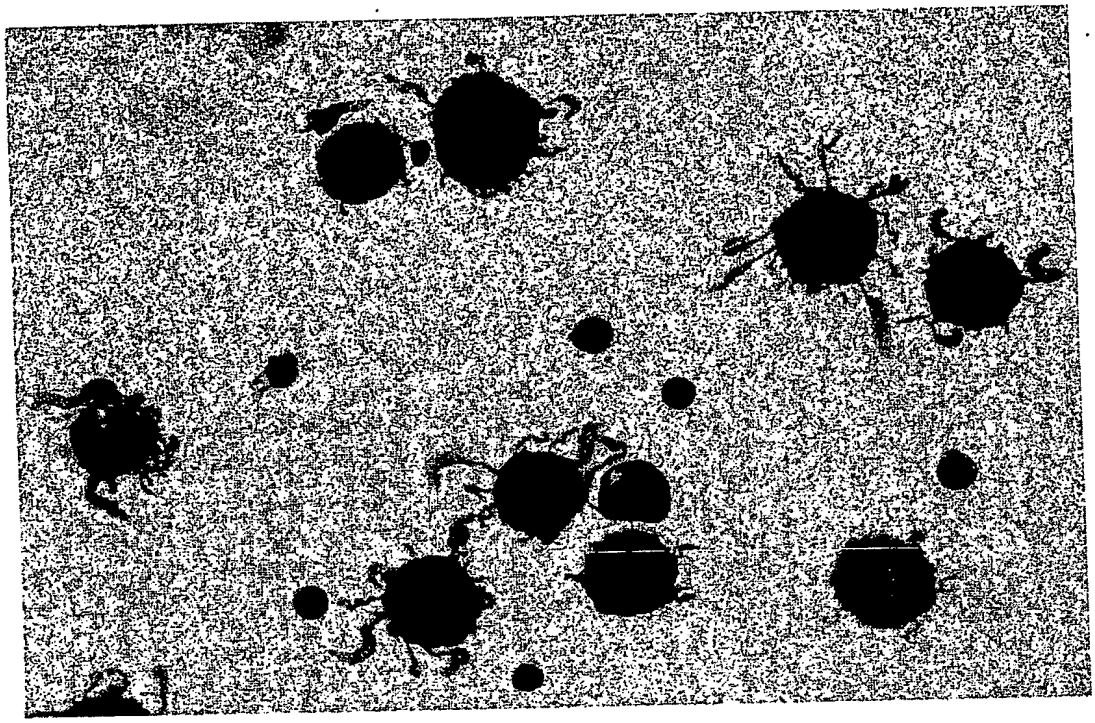

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Figure 3

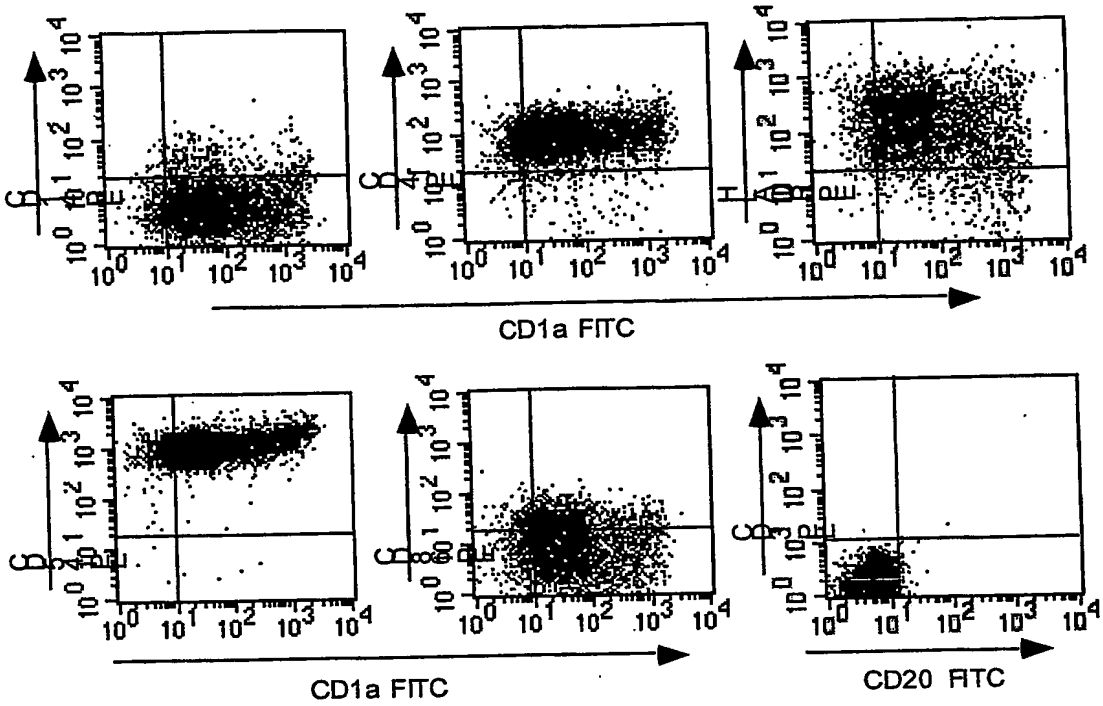

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Figure 4